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EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Performance Assessment and Sterility Verification of Prepared Media and Reagents

SOP Number: QC-11-03

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SOP Number: QC-11-03

Date Revised: 11-30-06

Initiated By: _____ Date: ___/___/___

Print Name:_____

Technical Review _____Date: ___/___/___

Print Name:_____
Technical Staff

QA Review: _____ Date: ___/___/___

Print Name:_____
QA Officer

Approved By: _____ Date: ___/___/___

Print Name:_____
Branch Chief

Effective Date: ___/___/___

Controlled Copy No.: _____

Withdrawn By: _____ Date: ___/___/___

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1.0 SCOPE AND APPLICATION:

1.1 This protocol describes quality control practices that will be performed on prepared media and reagents to assess the ability of the media to support recovery/growth of the test organisms and to verify media/reagent sterility.

2.0 DEFINITIONS:

2.1 General growth media = Media which support the growth of a broad range of organisms and are used to grow test cultures. General growth media are non-selective. Examples include nutrient agar and tryptic soy agar media.

2.2 Selective media = Media that permit the growth of one type of bacterium while inhibiting the growth of other types. This facilitates the isolation of a desired species. Examples include Mannitol Salt Agar, and Cetrimide Agar.

2.3 Differential media = Media that allows visual differentiation between two or more species of bacteria.

2.4 CFU = Colony Forming Unit

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in SOP MB-01, Biosafety in the Laboratory.

4.0 CAUTIONS: Not Applicable

5.0 INTERFERENCES: Not Applicable

5.1 All media and reagents must be labeled with appropriate control numbers (see SOP QC-09, Control Numbers) for tracking purposes. Incorrect labeling may interfere with the interpretation of results.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable about the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Inoculating loops

7.2 Incubator set at the appropriate temperature specified for the test conditions, organism, and media

7.3 Cultures of *Pseudomonas aeruginosa* (ATCC #15442) and *Staphylococcus aureus* (ATCC #6538)

7.4 Culture of *Mycobacterium bovis* (BCG) from Organon Teknika

7.5 Spore suspension of *Bacillus subtillis* (ATCC #19659); a commercial preparation of spores can be purchased. One source for the commercial preparation is Presque Isle Cultures, 3804 West Lake Rd., P.O. Box 8191, Erie PA 16505. In addition a *B. subtilis* spore suspension can be generated in-house as described in section 10.2.2.

7.6 Spore suspension of *Clostridium sporogenes* (ATCC # 3584) prepared as described in 10.2.4.

7.7 Pre-sterilized filtration unit with pore size of 0.45µm or 0.22µm such as Nalgene Analytical Filter Units.

8.0 INSTRUMENT OR METHOD CALIBRATION: Not applicable

9.0 SAMPLE HANDLING AND STORAGE: Not applicable

10.0 PROCEDURE AND ANALYSIS:

10.1 Media Performance Assessment: The performance verification of media should be conducted prior to use. If necessary, the performance tests may be conducted concurrently with use. Preferably, use the organism that corresponds to the anticipated use of the medium to determine media performance. Media controls used in neutralization confirmation studies may also be used in place of specific media performance tests.

10.2 Culture Preparation for Testing Solid and Liquid Media:

10.2.1 For *S. aureus*, and *P. aeruginosa*, inoculate tubes of nutrient broth or synthetic broth from a stock culture tube and incubate at 36°C ± 1°C for 24 ± 2, or use a 48-54 hour test culture per SOP-MB-05: AOAC Use Dilution Method for Testing Disinfectants.

10.2.2 For *B. subtilis*, a spore suspension is used. Prepare spore suspension as described in AOAC Official Method 966.04: Sporicidal Activity of Disinfectants – SOP MB-15. This procedure involves the use of nutrient agar amended with manganese sulfate as the sporulation medium. A spore suspension purchased from a vendor such as Presque Isle Cultures may also be utilized.

10.2.3 For *M. bovis* (BCG) inoculate Modified Proskauer Beck medium and incubate at 36°C ± 1° for 21-25 days. Prepare standardized culture with a 20% Transmittance (at 650 nm) as described in SOP MB-07, Confirmatory Tuberculocidal Method for Testing Disinfectant Efficacy, or use a suspension of *M. bovis* (BCG) as described in SOP MB-16, Quantitative Suspension Test Method for Determining Tuberculocidal Efficacy of Disinfectants Against *Mycobacterium bovis* (BCG), sections 10.1 and 10.2.

10.2.4 For *Clostridium sporogenes*, produce a spore suspension using cooked meat medium with manganese sulfate. Inoculate cooked meat medium with 5µg/mL manganese sulfate tubes with growth from a stock culture of *C. sporogenes* and incubate (aerobically) at 36°C ± 1°C for 72 ± 2 hours. Following incubation, filter the inoculum through moist glass wool to eliminate media pellets. After preparation of the spore suspension, analyze to determine the titer. The spore titer should be approximately 10^8 spores/mL. The filtered spore suspension is stored under refrigeration in screw cap tubes or bottles for no longer than 6 months.

10.3 **Inoculation of Solid Media (general growth media and selective media)**:

10.3.1 For each preparation of solid media in plates or tubes (e.g., tryptic soy agar, nutrient agar, M7H9), 6 plates will be reserved to assess the media's performance. Spread plate, in duplicate, three serial ten-fold dilutions of the test microbe.

- For media inoculated with *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *C. sporogenes*, plate 0.1 mL of the 10^{-5} to 10^{-7} dilutions. The dilutions plated assume an initial titer of approximately 10^8 to 10^9 CFU/mL.
- For media inoculated with *M. bovis*, plate 0.1 mL of the 10^{-4} to 10^{-6} dilutions of the standardized culture. The dilutions plated assume an initial titer of approximately 10^7 to 10^8 CFU/mL.
- Target counts of 30-300 CFU/plate are desirable and should result from at least one of the plated dilutions – this dilution will serve as the basis for determining media performance.

10.3.2 For solid media in tubes (e.g., nutrient agar slants, M7H9 slants), inoculate a minimum of 2 tubes per batch with an undiluted culture of the test microbe.

- For selective media in plates or tubes (e.g., Mannitol Salts Agar, Cetrimide Agar), streak (for plates) or stab (for tubes) inoculate a minimum of 2 tubes or plates with an undiluted culture of the appropriate target organism (i.e., the organism the media is designed to identify). An isolation streak should be performed on selective media in plates to aid in the assessment of the medium's reaction and organism's colony characteristics.

10.4 **Incubation of Solid Media**:

For solid media inoculated with *S. aureus, P. aeruginosa, B. subtilis* or *C. sporogenes*, incubate plates or tubes at 36°C ± 1°C for 24 to 48 hours. For *C. sporogenes*, incubate media under anaerobic conditions (e.g. Gas-Pak anaerobic environment generation system). For media inoculated with *M. bovis*, incubate plates or tubes at 36°C ± 1°C for 21-25 days.

10.5 **Performance Results for Solid Media:**

10.5.1 For plates, enumerate the colonies per plate, determine the average CFU/plate and CFU/mL of diluted inoculum, and assess the colony morphology (refer to SOP MB-02: Test Microbes for colony characteristics). Use plates exhibiting 30-300 CFU/plate for calculations. Record findings on form 16.1: Performance and Sterility Assessment of Media in Plates. Note: If the inoculum titer is below the target of 30-300, then either the media performance is unsatisfactory or the starting inoculum was substandard. In this case, the media performance assessment should be repeated. The final assessment is based on an assessment of both sets of data.

10.5.2 For tubes, record the presence or absence of growth. Record findings on the Performance and Sterility Assessment of Media in Tubes form (see 16.2). If growth occurs and exhibits typical morphology a "+" will be recorded. If no growth is apparent a "0" will be recorded. For atypical growth that is determined not to be a test microbe, record observations and indicate growth was a contaminant.

For selective media, verify the performance per the appropriate media reactions (e.g. agar turning fluorescent green) or colony characteristics, see SOP MB 02: Test Microbes. Complete the

form (see 16.2) under the "Performance Assessment" caption, by checking either Satisfactory or Unsatisfactory per the observations. For typical morphology and appearance, a "+" will be recorded. If no growth is apparent a "0" will be recorded. For growth that is determined not to be a test microbe, record observations and indicate growth was a contaminant. On the form under the "Performance Assessment" caption check either Satisfactory or Unsatisfactory per the observations.

10.6 **Inoculation of Liquid Media**:

10.6.1 For liquid media (e.g., letheen broth, nutrient broth, modified proskauer beck medium), 6 tubes for each batch will be kept aside for the performance testing. Inoculate tubes in duplicate with 0.1 ml aliqouts of three serial ten-fold dilutions of the appropriate culture, except for *M. bovis*, where an aliquot of 1.0 mL is used to inoculate tubes.

- For *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *C. sporogenes*, inoculum should be diluted to a level to allow for a challenge with low titer (a range of 1-50 CFU/tube)
- For *M. bovis* (BCG), a challenge of 1-50 CFU/tube is desirable for determining media performance.
- For *S. aureus*, *P. aeruginosa*, *B. subtilis*, and *C. sporogenes*, use 0.1 mL of the 10^{-5} to 10^{-7} dilutions.
- For *M. bovis*, use 1.0 mL of the 10^{-5} to 10^{-7} dilutions of the standardized culture.
- Verify CFU/tube by spread plating in duplicate on the appropriate medium – see section 10.3.1 for plating instructions.

10.7 **Incubation of Liquid Media**:

10.7.1 For liquid media inoculated with *S. aureus*, *P. aeruginosa*, *B. subtilis* or *C. sporogenes*, incubate at 36°C ± 1°C for 24 to 48 hours. For media inoculated with *M. bovis*, incubate at 36°C ± 1°C for 21 days, if no growth occurs at 21 days, incubation may be continued for up to 60 days. For liquid media inoculated with spores of *C. sporogenes*, incubate under aerobic conditions for 24 to 72 hours.

10.8 **Results for Liquid Media**:

10.8.1 Following incubation, the performance of each medium will be visually assessed and the observations will be recorded on form 16.2, Performance and Sterility Assessment of Media in Tubes. Plate counts will be read to determine the CFU/tube. For each tube, a "+" will be recorded if growth is observed (indicated by turbidity or growth) and a "0" will be recorded if growth is not observed. In the case of a contaminant, record observations and indicate growth was a contaminant.

10.8.2 Performance is judged to be satisfactory when at least one of the two tubes in a dilution set that received a sufficiently low challenge (1-50 CFU/tube) of the test microbe shows growth. The number of CFUs delivered to each tube in a set is based on the corresponding averaged plate counts for that dilution. All tubes in dilution sets receiving greater than the targeted challenge should show growth as well. Based on this criteria under the "Performance Assessment" caption on the form, check either Satisfactory or Unsatisfactory. Also record the performance assessment on the Media/Reagent Preparation Form (see SOP MB-10, Media and Reagents).

10.9 **Sterility Verification of Solid and Liquid Media**: Sterility verification must be performed on a minimum of 2% of each preparation of solid or liquid media.

10.9.1 Selected plates or tubes of media, for use with organisms other than *M. bovis* (BCG), may be placed directly in an incubator at 36°C ±1°C for 5-10 days. Perform for media purchased from a vendor as well.

10.9.2 Solid and liquid media used for the growth of *M. bovis* (BCG) are incubated at 36°C ± 1°C for 21-28 days.

10.9.3 Following incubation, if no growth on solid media or in liquid media is observed, record a "0" (satisfactory). If growth is observed, a "+" (unsatisfactory) is recorded on the appropriate Performance and Sterility Assessment form (see 16.0). On each form, fill-in "Sterility Assessment" by indicating either Satisfactory or Unsatisfactory per the observations. Also record the sterility assessment on the Media/Reagent Preparation Form (see SOP MB-10, Media and Reagents).

10.9.4 For solid media in plates, a satisfactory result for sterility is typically based on no growth observed on the plates following incubation. Although infrequent, an occasional bacterial or fungal colony may appear on the surface of the agar as a result of environmental contamination or poor aseptic technique when the plates were poured – this may not be indicative of poor media quality. In these instances, the remaining plates of the preparation in question should be examined prior to use to help determine the extent of the contamination, and an additional assessment of sterility should be performed. If any growth is observed in the second assessment, discard the medium.

10.10 **Sterility of Reagents (not used to support microbial growth)**:

10.10.1 If a reagent is dispensed in bottles, only one bottle per preparation is tested for sterility. Use a pre-sterilized 0.45µm or 0.22 µm filter unit and aseptically filter approximately 2% of the volume of each bottle of reagent. Pour approximately 2% of the volume into the pre-filtration reservoir. There are graduations on the reservoir that are sufficiently accurate for this purpose. Do not place anything such as a pipette into the reagent bottle. Because microbial contamination is expected to be sufficiently low or nonexistent, 2% of a preparation may be filtered using the same filter unit. Once filtration is complete, aseptically transfer the filter to a TSA plate and incubate for 5-10 days at 36°C ± 1°C.

10.10.2 For reagents that are dispensed in tubes, set aside the number of tubes that represents approximately 2% of the total volume of media prepared, filter the contents, plate the filter on TSA, and incubate for 5-10 days at 36°C ± 1°C.

- For example, if 1 liter of dilution water is prepared in 9 mL blanks, 2% of 1L would be 20 mL, so 3 tubes would be set aside.
- Using one pre-sterilized filtration unit, aseptically transfer the contents of all the tubes to the filtration reservoir and filter the contents. Once filtration is complete, transfer and incubate the filter on appropriate medium for 5-10 days at 36°C ± 1°C and assess filters for presence of microbial growth.

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0.10.3 Following incubation of filters, if no growth is observed, record a "0" (satisfactory). If growth is observed record a "+" (unsatisfactory). Record results on the Performance and Sterility Assessment form (see 16.0). In the case of reagents, record the assay results and observations on form 16.3, Sterility Verification for Reagents, as well as on the Media/Reagent Preparation and Performance Log Form (see SOP MB-10, Media and Reagents).

10.10.4 For testing the sterility of official carriers used in efficacy testing and research, perform the following:

- For stainless steel and porcelain carriers, place one carrier per autoclaved preparation into a 10 mL tube of Fluid Thioglycollate Medium (FTM) and incubate for 5-10 days at 36°C ± 1°C.
- For glass slide carriers place one carrier per autoclaved batch into a 20 mL tube of FTM and incubate for 5-10 days at 36°C ± 1°C.
- Following incubation, if no growth is observed, record a "0" and if growth is observed record a "+" on the appropriate form. Record the assay results and observations on form 16.4, Sterility Verification of Official Carriers, as well as on the Media/Reagent Preparation and Performance Log Form (see SOP MB-10, Media and Reagents).

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the appropriate forms. Completed forms are archived in notebooks kept in secured file cabinets in the file room D217. Only authorized personnel have access to the secured files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 The OPP Microbiology Laboratory conforms to 40CFR Part 160, Good Laboratory Practices. Appropriate quality control measures are integrated into each SOP.

13.2 For quality control purposes, the required information is documented on the appropriate record form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Any media/reagent failing (unsatisfactory) a growth assessment or sterility verification will be discarded.

14.2 When the media performance and sterility assessment are performed concurrently with the use of the media in testing and the media quality is deemed unsatisfactory, the specific use of the media in the test(s) should be investigated to determine the potential impact on the test results and the necessity for repeating the test(s). Testing must be repeated when unsatisfactory media and/or reagents are used for critical aspects of testing such as subculturing, confirmation testing, carrier counts and serial dilutions.

14.2.1 If the same preparation of media is available, it should be reevaluated for performance and/or sterility (failed component only) per this SOP. If the preparation is deemed satisfactory, it is not necessary to repeat the test(s).

14.2.2 If the unsatisfactory preparation is not available, investigate other sources of performance data such as test controls and neutralization tests. If additional data indicates satisfactory performance, repeating the test may not be necessary.

14.2.3 Document all findings on the appropriate performance and sterility forms.

15.0 REFERENCES: None

16.0 FORMS AND DATA SHEETS:

16.1 Performance and Sterility Assessment of Media in Plates

16.2 Performance and Sterility Assessment of Media in Tubes

16.3 Sterility Verification of Reagents

16.4 Sterility Verification of Official Carriers

Performance and Sterility Assessment of Media in Plates

OPP Microbiology Laboratory

Background and Preparation	
Media tested/Prep Number:	
Test organism/Control Number:	
Date Performed/Initials:	
Media used for enumeration:	☐ Yes ☐ No Fill in the appropriate Results block below
Total volume of media prepared:	
Number of plates analyzed for performance and sterility:	Performance: _____ Sterility: _____

Media Performance and Sterility Results for Plates **USED IN ENUMERATION**						
Performance: Plate Count Data Date/Initials:_____					Sterility Assessment† Date/Initials:_____	
Dilution	Plate 1	Plate 2	Avg. CFU/plate	Observations	Sterility (+/0)	Sterility (+/0)
					Plate# ___=___	Plate# ___=___
					Plate# ___=___	Plate# ___=___
					Plate# ___=___	Plate# ___=___
Avg. CFU/mL*:					Plate# ___=___	Plate# ___=___

Media Performance and Sterility Results for Plates **NOT USED IN ENUMERATION**†			
Performance: Date/Initials:_____		Sterility Assessment Date/Initials:_____	
Growth (+/0)		Sterility (+/0)	
Plate# ___=___	Plate# ___=___	Plate# ___=___	Plate# ___=___
Plate# ___=___	Plate# ___=___	Plate# ___=___	Plate# ___=___
Plate# ___=___	Plate# ___=___	Plate# ___=___	Plate# ___=___
Plate# ___=___	Plate# ___=___	Plate# ___=___	Plate# ___=___

† + = growth, 0 = no growth
*Based on plates in the range of 30-300 CFU/plate

Performance Assessment Satisfactory ☐ Unsatisfactory ☐
Sterility Assessment @ 36±1°C Satisfactory ☐ Unsatisfactory ☐

Performance and Sterility Assessment of Media in Tubes

OPP Microbiology Laboratory

Background and Preparation		
Media tested/Prep Number:		
Test organism/Control Number:		
Date performed/Initials:		
Media Type:	□ Solid	□ Liquid
Total volume of media prepared:		
Number of tubes analyzed for performance and sterility:	Performance: _____	Sterility: _____
Plating media/Prep Number:	□ N/A	

Results of Media Performance of Liquid Media in Tubes (36°C ± 1°C)				Date/Initials:_____		
Plate Counts				Performance Assessment at Each Dilution†		
Dilution	Plate 1	Plate 2	Avg. CFU/plate	Tube 1: (+/0)	Tube 2: (+/0)	Observations
Avg. CFU/mL*:						

Results of Sterility Assessment of **LIQUID MEDIA IN TUBES**	
Results Date/Initials:_____	
Growth (+/0)	Growth (+/0)
Tube# ___=___	Tube# ___=___
Tube# ___=___	Tube# ___=___
Tube# ___=___	Tube# ___=___
Tube# ___=___	Tube# ___=___

Sterility and Media Performance Results of **SOLID MEDIA IN TUBES**			
Performance† Date/Initials:_____		Sterility Assessment† Date/Initials:_____	
Tube# ___=___	Tube# ___=___	Tube# ___=___	Tube# ___=___
Tube# ___=___	Tube# ___=___	Tube# ___=___	Tube# ___=___
Tube# ___=___	Tube# ___=___	Tube# ___=___	Tube# ___=___
Tube# ___=___	Tube# ___=___	Tube# ___=___	Tube# ___=___

† + = growth, 0 = no growth
*Based on plates in the range of 30-300 CFU/plate

Performance Assessment	Satisfactory ☐	Unsatisfactory ☐
Sterility Assessment @ 36±1°C	Satisfactory ☐	Unsatisfactory ☐

Sterility Verification of Reagents
OPP Microbiology Laboratory

Background and Preparation	
Reagent tested/Prep Number:	
Date performed/Initials:	
Total volume of reagent prepared:	
Batch Volume Distribution Information: Indicate whether reagent was dispensed into bottles or tubes, the total number of bottles or tubes in the batch, the volume of each bottle or tube, and the volume that represents 2% of each bottle, or the number of tubes that represents 2% of the total volume dispensed into tubes.	□ Bottles\#_____ Volume/bottle_____ Volume analyzed per bottle:_____
	□ Tubes\#_____ Volume/tube_____ Total number of tubes analyzed:_____
Filter Unit	□ 0.22 µm filter unit □ 0.45 µm filter unit
Culture (plating) media/Prep Number:	

Results of Sterility Verification	
Results Date/Initials (36 ± 1°C):_____	
Incubation start date	
Incubation stop date	
Was growth observed?	□ Yes □ No
If Yes, how many colonies?	
Comments/Corrective Action:	

Additional Comments:

Sterility Assessment @ 36±1°C Satisfactory □ Unsatisfactory □

Sterility Verification of Official Carriers
OPP Microbiology Laboratory

Background and Preparation	
Carrier tested/Prep Number:	
Date performed/Initials:	
Type of carrier prepared:	□ Stainless Steel □ Porcelain □ Glass Slides □ Other
Total number of carriers prepared:	
Culture media/Prep Number:	FTM:

Results of Sterility Verification	
Results Date/Initials:_____	
Incubation start date	
Incubation stop date	
Was growth observed?	□ Yes □ No
If Yes, how many colonies?	
Comments/Corrective Action:	

Additional Comments:

Sterility Assessment @ 36±1°C Satisfactory □ Unsatisfactory □